Clin. exp. Immunol. (1992) **90**, 1–5

Phospholipid-containing toxic malaria antigens induce hypoglycaemia

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(Accepted for publication 22 June 1992)

SUMMARY

Hypoglycaemia is associated with severe malaria and is an important prognostic indicator. Molecules liberated during overnight incubation of erythrocytes infected with *Plasmodium yoelii* induce marked hypoglycaemia in normal mice, with a delayed time course compared with insulin; some, though weaker, activity could also be obtained by overnight incubation of uninfected erythrocytes. The active component shares many properties with the phospholipid-containing molecules which we have previously shown to be toxic and to induce the release of tumour necrosis factor (TNF) from macrophages. However a MoAb which neutralizes the cytotoxicity of tumour necrosis factor *in vitro* did not prevent this induction of hypoglycaemia, whereas antiserum against the toxic antigens did, as did immunization of normal (but not the immunoglobulin-deficient SCID) mice with the same material. Furthermore, normal mice injected with the antigens after immunization with phosphatidyl inositol or inositol monophosphate did not develop hypoglycaemia; the latter compound was also inhibitory when mixed with the antigens before injection. These compounds were previously shown to block the induction of TNF by the antigens and to induce the production of inhibitory antibodies. The role of these molecules in the etiology of the hypoglycaemia of malaria is discussed.

Keywords malaria toxic antigens hypoglycaemia phospholipids

INTRODUCTION

Hypoglycaemia is now recognized to be a major complication of severe *Plasmodium falciparum* malaria, particularly in children and pregnant women [1] and is associated with greatly increased mortality in patients with cerebral malaria [2,3]; it has also been demonstrated in severe rodent malaria [4]. Apart from cases proved to be due to hyperinsulinaemia following quinine therapy, there is no agreed mechanism to explain the hypoglycaemia: reduced levels of liver glycogen and gluconeogenesis, increased glucose consumption by both host and parasite, and even simple fasting, have all been proposed [1]. Recently a link has been established between severe malaria and raised plasma levels of tumour necrosis factor (TNF) [5–7], and Clark [8] has suggested that TNF overproduction may be responsible for a number of complications including hypoglycaemia. In our laboratory we have been attempting to identify the toxic molecules derived from parasitized erythrocytes (which we have previously referred to as exoantigens) that are responsible for triggering TNF release from macrophages. Our evidence suggests that they are heat-stable and resist digestion by proteases and that their TNF-inducing activity depends upon a phospholipid component [9]. Here we report that the same, or similar, molecules can induce severe hypoglycaemia in mice.

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MATERIALS AND METHODS

Mice

Outbred females at least 6 weeks old were normally used (Tuck No. 1; A.Tuck & Sons, Battlesbridge, UK). For immunization experiments, (C57 Bl X BALB/c) F1 or BALB/c mice (as controls for severe combined immunodeficient (SCID) mice) from the National Institute of Medical Research (London, UK) were used. Male SCID mice [10], also obtained from the National Institute of Medical Research, were housed in an isolator, provided with sterile food, water and bedding and used when 4 months old; before use they were screened to ensure they had no detectable circulating immunoglobulin.

Parasite supernatants

These were prepared as previously described [9] using the YM lethal variant of *P. yoelii*. Washed parasitized erythrocytes obtained from mice with more than 50% parasitaemia, as determined from blood films stained with Giemsa, were incubated in PBS (pH 7·3) at 10^8 parasitized cells/ml overnight on a roller at 37°C. Next day, supernatants were collected after

centrifugation at 500 *g* for 10 min, boiled for 5 min, centrifuged at 1300 *g*, passed through a 0·2 μm filter and stored at 4°C. Though we have previously referred to the active components of these supernatants as 'exoantigens' [9], we now prefer the term 'toxic antigens' because parasites lose their viability during the overnight incubation so that the antigens may not necessarily be released spontaneously. Control supernatants from normal erythrocytes were made in the same way.

Pronase digestion

Boiled supernatants were incubated for 24 h at 37°C in 10 μg/ml of pronase E (Sigma), boiled again, dialysed against PBS, then mixed with polymyxin B-agarose (Sigma) and centrifuged to remove any endotoxin, and sterilized by filtration. No protein was then detectable by BioRad assay (< 1 μg/ml).

Nitrous acid deamination

Pronase-digested supernatants were diluted in 0·16 M $NaNO_2$ in 25 mM sodium acetate at pH 3·5 and kept at room temperature for 5 h; they were then neutralized with NaOH.

Lipase digestion

Pronase-digested supernatants were incubated overnight at 37°C with 5 U/ml of lipase from wheat germ bound to agarose beads (Sigma). These were removed by centrifugation and the supernatant was then filtered.

Measurement of blood sugar

Glucose concentrations were determined from a drop of tail blood, using Glucostix and an Ames Glucometer (Miles Ltd, Stoke Poges, UK) according to the manufacturer's instructions. Observations on 243 normal outbred mice gave a mean of 7·64 ± 1·31 mmol/*l* (s.e.m. 0·08). Results are expressed as means ± s.d. of at least three groups of three mice, unless otherwise stated, and statistical differences were calculated by Student's *t*-test.

Immunization of mice

For antiserum against the toxic antigens, outbred mice were immunized with 0·2 ml of parasite supernatant intraperitoneally and bled 10–12 days later [11]. For antiserum against inositol monophosphate (IMP) (Sigma), mice were injected intraperitoneally with 200 μg per mouse and also bled after 12 days [12]. For immunization against phosphatidylinositol and IMP (C57Bl/BALB/c) F1 mice were used. PI was first deacylated by heating at 56°C for 2 h in 0·05 M NaOH, and then neutralized [9]. It was then diluted to 100 μg/ml in phosphate buffer pH 5·0 on ice and conjugated to 100 μg/ml of bovine serum albumin (BSA) (Sigma) by mixing with an equal volume of ice-cold 150 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC; Sigma) and leaving for 1 h. Excess EDC was then quenched by addition of lysine (Sigma) to 1 mg/ml. After dialysis, 0·5 ml of this material was injected intraperitoneally into a group of mice, at 2- or 3-week intervals. Lysine similarly conjugated to keyhole limpet haemocyanin (KLH) (Sigma) was used as a control antigen.

Antibody against TNF

A hamster MoAb against murine rTNF (TN3–19.12) made by Dr R. D. Schreiber [13] was kindly provided by Celltech Ltd, Slough. Mice were injected with 500 μg of antibody the day before and again 1 h before injection of parasite supernatants.

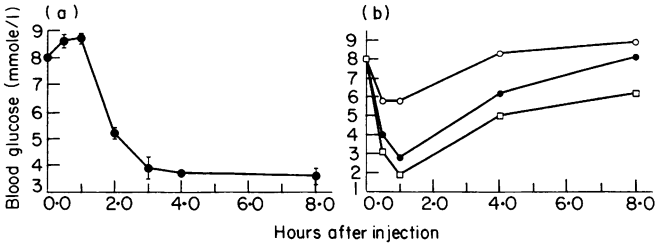

Fig. 1. Time course of the development of hypoglycaemia. (a) Parasite toxic antigens. Means (± s.e.m.) are shown for groups of at least 14 mice injected intraperitoneally with four different supernatants. (b) Insulin. Means of groups of three mice injected intraperitoneally with three different doses of human insulin. □, 0·8 U; ●, 0·2 U; ○, 0·05 U per mouse.

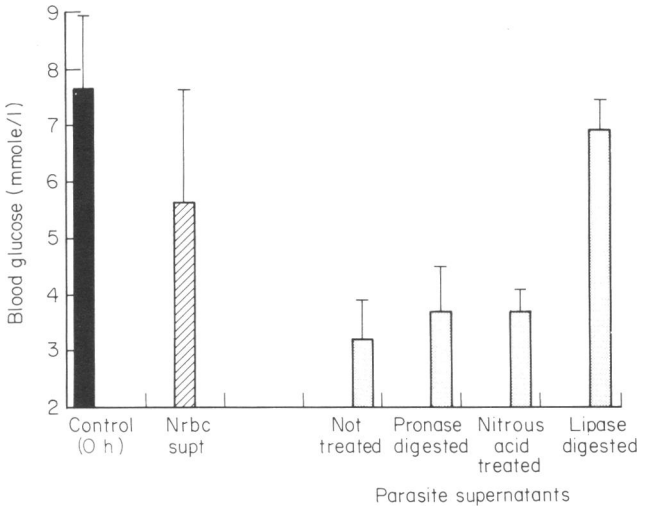

Fig. 2. Effect of various treatments of parasite supernatants on their induction of hypoglycaemia. Means ± s.d. of groups of mice before (■) and (▨, ▨) 4 h after i.p. injection of supernatants. Four different preparations of normal erythrocyte supernatants were tested and four parasite supernatants undigested, six after pronase digestion, two after deamination and two after lipase digestion. The s.e.m. of the mean was < 0·2 mmole/*l* for all groups. Nrbc supt, normal erythrocyte supernatant.

Other reagents

Lipopolysaccharide (LPS) was the phenol extract of *Escherichia coli* (055:B5) from Sigma and human insulin (Actrapid) was obtained from Nova.

RESULTS

During previous work we showed that mice made hypersensitive to TNF by administration of D-galactosamine were killed by injection of boiled supernatants from parasitized erythrocytes [14]. We also observed that even without D-galactosamine mice would sometimes die after injection of very active preparations, and those that survived frequently looked sick within a few hours, huddling together with ruffled fur, and sometimes developing diarrhoea. These exoantigen preparations, which are chemically and serologically distinguishable from LPS [9,14,15], usually induce the secretion from mouse peritoneal

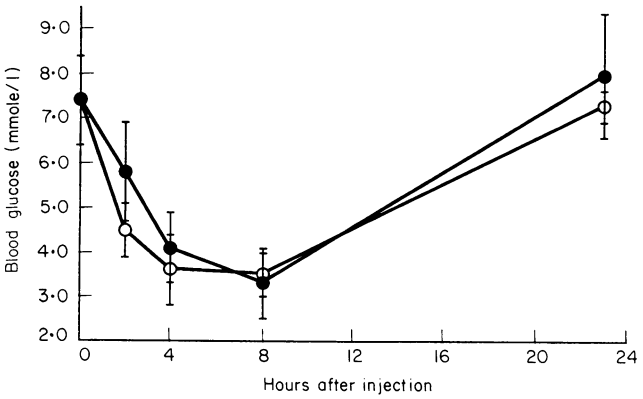

Fig. 3. Time course of the development of hypoglycaemia induced by parasite toxic antigens in the presence and absence of a MoAb against murine rTNF. Means ± s.d. from mice injected with 500 μg of antibody the day before injection of 0·5 ml of a pronase-digested supernatant. ○, Controls; ●, with antibody.

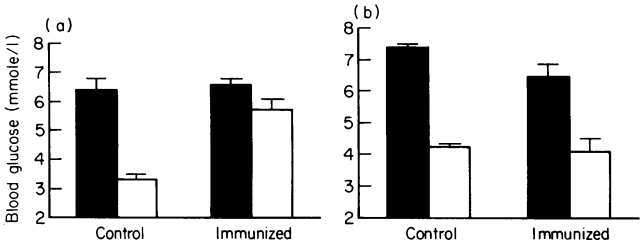

Fig. 4. Protection against induction of hypoglycaemia by immunization with the antigens. (a) Normal mice. (b) SCID mice. ■, 0 h; □, 4 h readings. Pooled results (means ± s.e.m.) from mice immunized by one or two injections of 0·2 mg of a freeze-dried parasite supernatant and then challenged 9 or 13 days after the last injection with the same supernatant. The results shown are from two experiments pooled for the normal mice and one with the SCID mice. The differences between 0 and 4 h observations were significantly different in all cases ($P < 0{\cdot}0001$) except for the group of immunized normal mice.

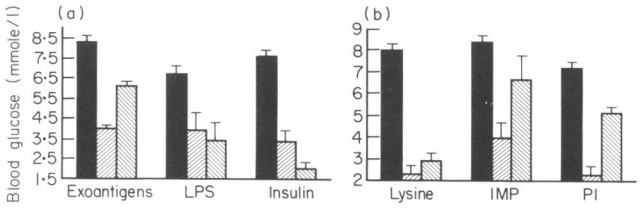

Fig. 5. Inhibition by IMP, and by immunization with IMP or PI, of induction of hypoglycaemia by parasite antigens. (a) Inhibitory effect of inositol monophosphate (IMP) on parasite antigens, compared with lipopolysaccharide (LPS) and insulin. ■, 0 h; ▨, 4 h after i.p. injection of 0·5 ml of supernatant or 10 μg of LPS, or 1 h after injection of 0·2 U of insulin; ▧, same after mixing stimulant with 1 mg of IMP per mouse. Means ± s.d. of three groups of three mice given three different antigen preparations are shown, compared with two groups of three mice given LPS and a group of five mice given insulin. The 4 h readings for antigens in the presence and absence of IMP were significantly different ($P < 0{\cdot}0001$). (b) Protection against induction of hypoglycaemia by immunization of mice with IMP or phosphatidyl inositol (PI), compared with an unrelated antigen. ■, 0 h; ▨, control unimmunized mice; ▧, immunized mice, both 4 h after challenge with the same antigen preparations. Means ± s.d. from a group of five mice immunized with 200 μg of IMP 12 days before challenge, and groups of three mice that had received three injections of PI conjugated to bovine serum albumin (BSA) or of lysine conjugated to KLH before challenge.

macrophages of as much TNF as about 1 μg/ml of LPS [14]. In preliminary experiments, measurement of the blood glucose of a group of six mice injected intraperitoneally with 0·5 ml of such an exoantigen-containing supernatant showed a decrease 4 h later from a mean ± s.d. of 6·75 ± 1·2 to 3·45 ± 0·4 mmol/*l*; similarly, another supernatant caused a drop from a mean ± s.d. of 7·14 ± 1·4 to 3·4 ± 0·6 mmol/*l* when 0·2 ml, 0·4 ml or 1 ml was injected ($P < 0{\cdot}0001$ in both cases). No significant changes in blood glucose levels were ever observed in mice injected with PBS. As reported by others [16,17], we found that injection of LPS (10 μg per mouse) caused a similar drop in blood glucose, to a mean ± s.d. of 2·9 ± 1·1 in 13 mice at 4 h.

Time course of development of hypoglycaemia

Blood glucose measurements made at intervals after injection of different supernatants showed that no significant decrease, and often a slight increase, occurred during the first hour but that the level dropped by 2 h, reached its lowest point between 4–8 h and then returned more or less to normal by 22 h (to a mean ± s.d. of 6·4 ± 1·3). Figure 1 shows the pooled results obtained with four different supernatants, and shows a completely different pattern from readings obtained from mice given three different concentrations of human insulin. All the doses of insulin tested caused a maximum decrease 1 h after injection which, in contrast to findings with the parasite supernatants, then rose again by 4 h; with the concentration (0·2 U per mouse) which induced hypoglycaemia of roughly similar intensity to the supernatants — and even with the highest dose—it had returned to levels within the normal range by 8 h.

In subsequent experiments, blood glucose was measured routinely before and 4 h after injection of 0·2–0·5 ml of supernatants, with occasional checks at 2, 6 or 8 h. Levels below 2·5 mmol/*l* were rare. There was some variation between individual mice, both before and after injection of supernatants, and analysis of pooled results from repeated experiments showed that the s.d. of the mean, whether before or after injection, was always about 1 mmole/*l*.

Nature of the hypoglycaemia-inducing component of parasite supernatants

Supernatants made from uninfected erythrocytes sometimes induced significant hypoglycaemia but this was significantly less ($P < 0{\cdot}0001$) than that induced by supernatants derived from the same number of parasitized cells (Fig. 2). Indeed, preliminary experiments suggested that supernatants from parasitized erythrocytes were at least 10 times more active. Since we had found that the TNF-inducing activity of parasite toxic antigens *in vitro* was greatly increased by digestion with proteases [9], experiments were done to see if supernatants treated with pronase were also more potent in their effect on blood glucose. However, pooled results obtained with six different preparations showed that the hypoglycaemia induced was not significantly different from undigested preparations (Fig. 2). We had previously shown that the TNF-inducing activity of the antigens was unaffected by deamination by treatment with HNO_2 (in contrast to glycosylphosphatidylinositol (GPI) anchors of parasite anti-

gens which are known to be disrupted by such treatment) [9]. Deamination of pronase-digested supernatants did not abolish their ability to cause hypoglycaemia, but digestion by a lipase did (Fig. 2), as it did the TNF-inducing capacity [9].

The role of TNF

Recombinant TNF (rTNF) has been shown to cause a significant decrease in blood levels of glucose 4 h after administration [16]. To see whether TNF elicited by parasite toxic antigens was responsible for the hypoglycaemia induced by the supernatants, mice given two injections of a neutralizing MoAb against murine rTNF were then injected with pronase-digested exoantigen preparations. In four different experiments the mean blood glucose after 4 h never dropped quite as much as in the controls, but the time course was unaffected (Fig. 3) and the differences from the control at 4 h were never statistically significant ($P < 0{\cdot}03$; the mean for pooled results from mice given antibody was $4{\cdot}1 \pm 0{\cdot}75$, compared with $3{\cdot}7 \pm 0{\cdot}78$ for controls). Like others [17], we also found that antibody against TNF did not prevent the induction of hypoglycaemia by LPS (data not shown).

Immunization against hypoglycaemia

Mice immunized 9–12 days earlier with a parasite supernatant are protected against the toxic effects of the antigens [14]. Similarly, mice that had been immunized by one or two injections of a parasite supernatant did not show the same drop in blood glucose 4 h after subsequent challenge with the supernatant as did control mice (Fig. 4a). That this was probably mediated by antibody is suggested by the lack of protection observed in SCID mice immunized similarly by the same antigen preparation (Fig. 4b).

Serum from mice immunized by injection of supernatants 10–12 days earlier contains antibody (mainly IgM) which inhibits the production of TNF by the toxic antigens [11]. A similar antiserum when mixed with a supernatant (1:5) before injection into a group of mice prevented the development of hypoglycaemia 4 h later: their mean blood glucose was $6{\cdot}8 \pm 1{\cdot}25$ compared with $3{\cdot}1 \pm 1{\cdot}1$ mmole/*l* for mice given supernatant mixed with normal mouse serum ($P < 0{\cdot}01$).

Inhibition by IMP and by immunization with IMP or PI

In previously described experiments in which we investigated the effect of various phospholipids on TNF induction by the antigens *in vitro*, phosphatidylinositol (PI) and the related compound IMP were shown to block this activity effectively, presumably by competitive inhibition with a receptor [15]. We found that injection of IMP (1 mg/mouse) mixed with pronase-digested supernatants also significantly reduced their induction of hypoglycaemia. This concentration of IMP had no effect on either the degree or the time course of the hypoglycaemia induced by insulin or by 10 μg of LPS: the 4 h readings are illustrated (Fig. 5a). Furthermore, antiserum made against IMP, which blocks the induction of TNF by the parasite phospholipid antigens [12], also inhibited the induction of hypoglycaemia when mixed with the antigens to give a 1:20 final dilution before injection (mean $\pm$ s.d. at 4 h of $5{\cdot}9 \pm 0{\cdot}17$ compared with a control of $3{\cdot}8 \pm 0{\cdot}1$ mmole/*l* ($P < 0{\cdot}0001$)).

Mice immunized with IMP or with PI (conjugated to BSA) also failed to develop hypoglycaemia when challenged with a parasite supernatant, whereas control mice immunized similarly with lysine conjugated to KLH responded like normal unimmunized mice (Fig. 5b).

We conclude that the molecule(s) which induce hypoglycaemia can behave as antigens, giving rise to protective antibodies, and that their active component contains PI.

DISCUSSION

The essential finding here is that a product of erythrocytes infected with malaria parasites incubated *in vitro* can induce hypoglycaemia in uninfected mice. Non-parasitized erythrocytes produce a similar but weaker effect. This may suggest that the active molecule is one present in normal erythrocytes that is perhaps increased in amount or accessibility by the parasite. On the available evidence there is a strong resemblance to the phospholipids present in supernatants which induce the production of TNF *in vitro* and *in vivo* and which are toxic to mice [9,14]. Thus both TNF and hypoglycaemia-inducing activities appear to be associated with a lipid component, but not protein, and to act in a way that can be inhibited by IMP [15] and by antibody against IMP and PI [12]. It may be relevant that several erythrocyte lipids have been shown to alter their distribution in the membrane of malaria-infected erythrocytes [18]. The lack of effect of IMP on hypoglycaemia induced by LPS indicates that there was no important contamination of our samples by endotoxin. The fact that protease digestion did not enhance the hypoglycaemic effect of parasite supernatants, whereas it significantly increased the amount of TNF they induced *in vitro* [9], may perhaps be explained by the tendency of phospholipids to associate with proteins, which would presumably happen readily *in vivo*. Indeed we have found that the TNF-inducing activity of protein-free preparations is reduced or lost if they are mixed with delipidated serum albumin but can be recovered if they are then treated with pronase (Bate, unpublished work).

Several parasite antigens such as the merozoite surface antigen MSP-1 [19] are known to be anchored to the membrane by PI-containing structures which form an essential part of the GPI anchor that attaches some proteins to surface membranes (reviewed in [20]). At least 10 different GPI lipids have been reported to be present in *P. falciparum* culture supernatants [21] and it seems that TNF and hypoglycaemia-inducing molecules are likely to be among them. Further experiments with purified material are necessary to determine whether or not the same molecule can induce both effects. The lack of any significant inhibition by antibody against TNF does not answer this question: antibody against TNF also failed to prevent LPS-induced hypoglycaemia [17], suggesting that TNF is not the principal mediator, although it is possible that the antibody fails to reach and neutralize cytokine produced by one cell before it acts on an adjacent cell. IL-1 has also been shown to cause some of the fall in blood glucose levels induced by LPS, since an IL-1 receptor antagonist partially blocked the LPS effect, although it did not diminish hypoglycaemia induced by rTNF [22]. Injection of rIL-1 into mice has been reported to cause hypoglycaemia and raise levels of insulin [23], but in preliminary experiments we found that serum taken from mice with exoantigen-induced hypoglycaemia did not contain more insulin than normal mouse serum (unpublished work with P. Holloway). Another possibility is that the toxic antigens might act directly to alter glucose utilization without the mediation of any cytokines, especially since the putative insulin second

messenger is also believed to contain a phosphatidylinositol glycan structure and IMP has been shown to block the effect of insulin on lipogenesis [24]; this possibility is being investigated using adipocytes *in vitro*, in collaboration with Professor E. D. Saggerson and Dr T. W. Rademacher.

Since molecules are released from parasitized erythrocytes, *in vitro* at least, which can induce hypoglycaemia in normal mice, it is surprising that we found no significant change in levels of blood glucose during infection with the lethal *P. yoelii*, except on the day before death when the mice are already moribund, as has also been reported for rats infected with *P. berghei* [4], and not at all with the non-lethal *P. yoelii*. Yet the mice must be exposed to these molecules since they develop antibodies that block TNF induction by the phospholipid antigens at the time they begin to recover from the non-lethal infection (unpublished results). Either the antibodies are sufficient to prevent hypoglycaemia, even early in infection when titres are very low, or there are other blocking factors in serum. One such molecule might be β-2 glycoprotein 1 (apolipoprotein H) which binds to some phospholipids and which might therefore block some of their pathological effects [25], except when it is overcome by excess levels. Protection against some of the severe complications of malaria might be another important role for this intriguing molecule in addition to its regulatory role in blood coagulation [26]. It would be interesting to test whether genetically determined lower levels of β-2 glycoprotein 1 [27] are related to susceptibility in the 2% of children who develop severe malaria [28].

ACKNOWLEDGMENTS

We would like to thank Professor E.D. Saggerson and Dr T.W. Rademacher for helpful discussions, Dr R.D. Schreiber and Celltech Ltd for providing us with antibody against murine TNF and Ms Claire Robert for help with some of the assays. This work was supported by the Medical Research Council and the Science and Technology for Development Programme of the European Community (Contract No. TS2-0277-C).

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